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DESCRIPTION

INTRAVENOUS NANOPARTICLES FOR TARGETING DRUG DELIVERY
AND SUSTAINED DRUG RELEASE

TECHNICAL FIELD

The present invention relates to intravenous nanoparticles encapsulating low-molecular weight, water-soluble and non-peptide drugs that are intended for the purposes of targeting drug delivery and sustained drug release. The invention also relates to a production method of such nanoparticles. Specifically, the present invention relates to intravenous nanoparticles which can deliver low-molecular weight, water-soluble and non-peptide drugs to target lesion site where the particles gradually release the drugs over a prolonged period of time, and a production method thereof. Hereon, intravenous nanoparticles mean nanoparticles for intravenous administration containing drugs.

BACKGROUND ART

Many researchers have developed and proposed poly(lactic-co-glycolic acid) (PLGA) or poly(lactic acid) (PLA) microparticles and nanoparticles that encapsulate low-molecular weight, water-soluble drugs.

For example, US patent No. 4,652,441 describes PLGA microcapsules containing physiologically active polypeptides and a production method thereof. Japanese National Publication No. Hei 10-511957 describes PLGA nanoparticles for intravascular administration containing various therapeutic agents. Also, Japanese Patent Laid-Open Publication No. Hei 8-217691 discloses a sustained-release formulation comprising PLGA microcapsules encapsulating physiologically active, water-soluble peptide compounds, which were prepared in the form of water-insoluble or hardly water-soluble polyvalent metal salts.

However, none of these patent publications mention or suggest the concept of hydrophobicizing a low-molecular weight, water-soluble and non-peptide drug with the use of metal ions prior to the encapsulation of the drug into nanoparticles so as to make
5   intravenous nanoparticles suitable for the targeting delivery and sustained release of drugs.

The present inventors also have filed patent applications (e.g., Japanese Patent Application No. 2002-159190) concerning formulations comprising poly(lactic-co-glycolic acid) (PLGA) or
10   poly(lactic acid) (PLA) nanoparticles. The nanoparticles suggested by the present inventors, however, could only offer a low encapsulation efficiency of the low-molecular weight, water-soluble drugs. Attempts were therefore made to increase the hydrophobicity and thereby the encapsulation rate of the drug through processes
15   including esterification. However, such attempts resulted in a decrease in the length of time over which the nanoparticles can release the encapsulated drug, though the encapsulation rate was improved to some extent. In other words, the desired sustained drug-releasing property of the nanoparticles was compromised in these
20   approaches.

Accordingly, it is an objective of the present invention to provide intravenous nanoparticles encapsulating a low-molecular weight, water-soluble and non-peptide drug that are capable of
25   targeting a specific lesion site and are less likely to burst at an early stage of administration so that they can gradually release the drug at the lesion site over a prolonged period of time.

It is another objective of the present invention to provide a simple method for preparing such intravenous nanoparticles that
30   enables large-scale production of the product.

In an effort to attain the above-described goals, the present inventors drew attention to the fact that low-molecular weight, water-soluble and non-peptide drugs interact with certain metal ions.

Specifically, the present inventors have examined the possibility of
allowing such low-molecular weight, water-soluble and non-peptide
drugs to bind to metal ion to impart a hydrophobicity to the drugs,
thereby facilitating encapsulation of the drugs into PLGA or PLA

5 nanoparticles. As a result, the present inventors have discovered
that such drugs, when bound to a metal ion, become hydrophobic and
thus can be readily encapsulated in PLGA or PLA nanoparticles. In
fact, it has proven that the encapsulation efficiency of the
incorporation of the hydrophobicized drugs into the nanoparticles

10 was extremely high. The present inventors have also discovered that
the nanoparticles so produced have an ability to gradually release
the drugs over time and tend to accumulate specific lesion sites in
living body. This implied the possibility that such nanoparticles
can be suitably used in designing a formulation for effective

15 targeted drug delivery and sustained drug release. The discoveries
ultimately led the present inventors to devise the present invention.

DISCLOSURE OF THE INVENTION
        Accordingly, one aspect of the present invention concerns

20 intravenous nanoparticles designed for targeting drug delivery and
sustained drug release. The nanoparticles are characterized in that
a low-molecular weight, water-soluble and non-peptide drug is made
hydrophobic by a metal ion and is encapsulated in nanoparticles
formed of poly(lactic-co-glycolic acid) (PLGA) or poly(lactic acid)

25 (PLA), and a surfactant is applied to the surface of the PLGA or PLA
nanoparticles.
        In one specific embodiment of the intravenous nanoparticles
according to the present invention, the PLGA or PLA nanoparticles
has a diameter of 50 to 300 nm.

30   In one specific embodiment of the intravenous nanoparticles
according to the present invention, the low-molecular weight, water-
soluble and non-peptide drug to be encapsulated in the PLGA or PLA
nanoparticles has a molecular weight of 1000 or lower.

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In another specific embodiment of the intravenous nanoparticles according to the present invention, the metal ion to be bound to the low-molecular weight, water-soluble and non-peptide drug is any of zinc, iron, copper, nickel, beryllium, manganese, and

5   cobalt.

In a further specific embodiment of the intravenous nanoparticles according to the present invention, the low-molecular weight, water-soluble and non-peptide drug to be encapsulated in the PLGA or PLA nanoparticles has a phosphate group or a carboxyl group

10   in its molecule.

In a still more specific embodiment of the intravenous nanoparticles according to the present invention, the low-molecular weight, water-soluble and non-peptide drug is a steroidal anti-inflammatory agent, a non-steroidal anti-inflammatory agent, a

15   prostanoid, an antimicrobial agent, or an anticancer agent.

In a still more specific embodiment of the intravenous nanoparticles, the surfactant to coat the surface of the PLGA or PLA nanoparticles encapsulating the low-molecular weight, water-soluble and non-peptide drug is a polyoxyethylene polyoxypropylene glycol, a

20   polysorbate, a polyoxyethylene octylphenyl ether, a lecithin, or a polyvinylalcohol.

Another aspect of the present invention concerns a method for producing intravenous nanoparticles for targeting drug delivery and sustained drug release. Specifically, the method comprises the steps

25   of hydrophobicizing a low-molecular weight, water-soluble and non-peptide drug by the use of metal ion; dissolving or suspending, along with PLGA or PLA, the low-molecular weight, non-peptide drug in a water-miscible organic solvent; and adding the resulting solution or the suspension to an aqueous solution of a surfactant to

30   apply the surfactant to the surface of the PLGA or PLA nanoparticles.

In one specific embodiment of the method for producing intravenous nanoparticles according to the present invention, the resulting PLGA or PLA particles have a diameter 50 to 300nm.

In another specific embodiment of the method for producing intravenous nanoparticles according to the present invention, the low-molecular weight, water-soluble and non-peptide drug to be encapsulated in the PLGA or PLA nanoparticles has a molecular weight of 1000 or lower.

In a further specific embodiment of the method for producing intravenous nanoparticles according to the present invention, the metal ion to be bound to the low-molecular weight, water-soluble and non-peptide drug is any of zinc, iron, copper, nickel, beryllium, manganese, and cobalt.

In a still more specific embodiment of the method for producing intravenous nanoparticles according to the present invention, the low-molecular weight, water-soluble and non-peptide drug to be encapsulated in the PLGA or PLA nanoparticles has a phosphate group or a carboxyl group in its molecule.

In a still more specific embodiment of the method for producing intravenous nanoparticles according to the present invention, the low-molecular weight, water-soluble and non-peptide drug is a steroidal anti-inflammatory agent, a non-steroidal anti-inflammatory agent, a prostanoid, an antimicrobial agent, or an anticancer agent.

In a still more specific embodiment of the method for producing intravenous nanoparticles according to the present invention, the surfactant to coat the surface of the PLGA or PLA nanoparticles encapsulating the low-molecular weight, water-soluble and non-peptide drug is a polyoxyethylene polyoxypropylene glycol, a polysorbate, a polyoxyethylene octylphenyl ether, a lecithin, or a polyvinylalcohol.

Another aspect of the present invention concerns a therapeutic preparation containing as an active ingredient the above-described nanoparticles. Specifically, the therapeutic preparation is an anti-inflammatory/anti-rheumatoid agent containing as an active ingredient the nanoparticles encapsulating a water-soluble steroid.

BEST MODE FOR CARRYING OUT THE INVENTION

As described above, the present invention comprises
biodegradable PLGA or PLA nanoparticles; a low-molecular weight,
water-soluble and non-peptide drug bound to a metal ion and
encapsulated in the nanoparticles; and a surfactant applied to the
surfaces of the nanoparticles.

Specifically, the intravenous nanoparticles of the present
invention designed for targeting drug delivery and sustained drug
release comprise a low-molecular weight, water-soluble and non-
peptide drug that has been hydrophobicized with a metal ion and has
been encapsulated in PLGA or PLA nanoparticles with a surfactant
subsequently applied to their surfaces.

In this regard, it has been found that the nanoparticles of
the present invention are most effectively uptaken by the target
lesion site when they have a diameter of 50 to 300nm. The
nanoparticles having a diameter less than 50nm tend to be uptaken by
regions other than the intended lesion sites and are therefore
undesirable, as are the nanoparticles having a diameter larger than
300nm, which tend to be uptaken by endothelial cells.

One characteristic feature of the present invention is that
the low-molecular weight, water-soluble and non-peptide drug is
bound to a metal ion so that the low-molecular weight drug will
become hydrophobic and is thus effectively encapsulated in the
nanoparticles. Among the metal ions suitable for this purpose are
zinc ion, iron ion, copper ion, nickel ion, beryllium ion, manganese
ion, and cobalt ion. Of these, zinc ion and iron ion are
particularly preferred.

For this reason, the low-molecular weight, water-soluble and
non-peptide drug to be encapsulated in the PLGA or PLA nanoparticles
in accordance with the present invention preferably includes a
phosphate group or a carboxyl group in its molecule so that the drug

can readily bind to the metal ion to become hydrophobic.

Preferably, the low-molecular weight, water-soluble and non-peptide drug has a molecular weight of 1000 or less.

While various drugs can be used as the low-molecular weight,
water-soluble and non-peptide drug in the present invention,
particularly preferred are water-soluble steroidal anti-inflammatory
agents, non-steroidal anti-inflammatory agents, prostanoids,
antimicrobial agents, and anticancer agents.

Specific examples of steroidal anti-inflammatory agents
include betamethasone phosphate, dexamethasone phosphate,
prednisolone phosphate, hydrocortisone phosphate, prednisolone
succinate, and hydrocortisone succinate.

Examples of non-steroidal anti-inflammatory agents include
loxoprofen sodium, and diclofenac sodium.

Examples of prostanoids include Prostaglandin E_1 (PGE$_1$), while
examples of antimicrobial agents include vancomycin, chloramphenicol
succinate, latamoxef, cefpirome, clindamycin phosphate, and
carumonam. Examples of anticancer agents include, but are not
limited to, vincristin, and vinblastine.

In one exemplary process of the present invention, the
intravenous nanoparticles are produced in the following manner: The
low-molecular weight, water-soluble and non-peptide drug is first
bound to the metal ion to make the agent hydrophobic. The drug is
then dissolved or suspended, along with PLGA or PLA, in a water-
miscible organic solvent. The resulting solution or suspension is
added to an aqueous solution of a surfactant and the mixture is
stirred to obtain the desired nanoparticles.

Examples of the water-miscible organic solvents for use in the
present invention include, but are not limited to, acetone,
acetonitrile, ethanol, methanol, propanol, dimethylformamide,
dimethylsulfoxide, dioxane, and mixtures thereof.

Examples of the surfactants include polyoxyethylene
polyoxypropylene glycols, polysorbates, polyoxyethylene octylphenyl

ethers, lecithin, and polyvinylalcohol.

Preferably, the nanoparticles of the present invention so
produced are purified by centrifugation, gel filtration, fiber
dialysis, or ultrafiltration and are subsequently freeze-dried for
5   storage to ensure the stability of PLGA or PLA as ingredient.

Upon freeze-drying, a stabilizing agent and an isotonizing
agent are preferably added to the nanoparticles suspension so that
the freeze-dried preparation can be resuspended for administration.
Preferred examples of the stabilizing agent and isotonizing agent
10   include sucrose and trehalose, which are preferably added in an
amount (by weight) 5 times or greater than the amount of the
nanoparticles.

The nanoparticles prepared in the above-described manner are
intravenously administered to target various inflammatory sites,
15   vascular lesions, infected sites, and malignant tumor tissues where
the particles effectively accumulate and sustainedly release the
encapsulated low-molecular weight, water-soluble and non-peptide
drug over time to provide the desired biological activities for a
prolonged period of time. This is where another advantageous feature
20   of the nanoparticles of the present invention comes in: the metal
ion acts to prevent the encapsulated low-molecular weight, water-
soluble and non-peptide drug from bursting release out of the
nanoparticles at an early stage after administration, thereby
allowing the sustained release of the drug for a prolonged period of
25   time.

Thus, in order for the nanoparticles to be usable as a medical
formulation, it is important to control, depending on the intended
purposes, the surface properties and the particle size of the
nanoparticles, as well as the encapsulation rate and the release
30   profile of the low-molecular weight, water-soluble and non-peptide
drug. For instance, the surface properties of the nanoparticles can
be controlled by using different types of surfactants.

Adjusting the particle size of the nanoparticles is important

also because the distribution of the nanoparticles within living body is strongly influenced by the particle size. To this end, the size of the nanoparticles is adjusted by taking into account how well the particles accumulate to different lesion sites (e.g.,

5   inflammatory sites, vascular lesion sites, infected sites, and malignant tumor tissues). Specifically, the particle size can be adjusted by controlling the conditions during the preparation of the nanoparticles, including the rate at which the aqueous phase is stirred, the amount of the organic solvent used, and the rate at

10   which the organic solvent is added to aqueous phase.

The efficiency of encapsulation of the low-molecular weight, water-soluble and non-peptide drug into the PLGA or PLA nanoparticles largely depends on the physical properties of the low-molecular weight drug. In general, hydrophilic (water-soluble) drugs

15   tend to be incorporated into the PLGA or PLA nanoparticles less efficiently than hydrophobic drugs. For this reason, the low-molecular weight, water-soluble and non-peptide drug for use in the present invention needs to be bound to a metal ion to impart a hydrophobicity to the agent. Specifically, this is done by allowing

20   the low-molecular weight, water-soluble and non-peptide drug to bind to a metal ion in such a manner that the drug forms water-insoluble precipitates.

For that purpose, such functional groups as phosphate and carboxyl, which are capable of binding to the metal ion, are

25   preferably introduced into the molecules of the low-molecular weight, water-soluble and non-peptide drug. It is also required that any functional groups present in the drug molecules that do not participate in, or interrupt, the formation of the precipitation with the metal ion must be protected with proper protective groups.

30   Furthermore, the type and amount of the organic solvent used and the rate at which the organic solvent is poured also affect the particle size of the nanoparticles and therefore need to be optimized.

With respect to the PLGA and PLA that serve as ingredient of
the nanoparticles, PLGA or PLA with different molecular weights may
be used to adjust the rate at which the encapsulated low-molecular
weight, water-soluble and non-peptide drug is released from the
5   nanoparticles.

To evaluate the nanoparticles of the present invention, it is
essential to construct *in vitro* or animal (*in vivo*) models suitable
for the evaluation of PK/PD (pharmacokinetics/pharmacodynamics) of
the nanoparticles.

10   As described above, the present invention has achieved a high
encapsulation rate of the low-molecular weight, water-soluble and
non-peptide drug into the PLGA or PLA nanoparticles by the use of
metal ions to impart a hydrophobicity to the drug. The present
invention allows the simple, industrial-scale production of the
15   intravenous nanoparticles designed for the purpose of targeting drug
delivery to target lesion sites where the particles can gradually
release the drug over a prolonged period of time.

EXAMPLES

20   The present invention will now be described in detail with
reference to examples and test examples.

Example 1: Formation of water-insoluble precipitates of low-
molecular weight, water-soluble and non-peptide drug with metal ion

25   Compounds shown in Table 1 below were used to as the low-
molecular weight, water-soluble and non-peptide drug having
phosphate groups. Each compound was dissolved in a 0.2M Tris-HCl
buffer solution (pH7.8) to a concentration of 20mM. The solution was
then added to equal volume of 100mM aqueous solutions of different
30   metal ions. The turbidity of each of the resulting mixtures was
observed.

The results are shown in Table 1 below.

Table 1: Formation of precipitates of low-molecular weight, water-soluble and non-peptide drugs with metal ions

		Low-molecular weight, water-soluble and non-peptide drugs				
		Naphthyl-phosphate	betamethasone phosphate	Dexamethasone phosphate	riboflavin phosphate	Tris-HCl buffer solution (0.1M/pH7.8)
Metal ions	NiCl$_2$	–	–	+	–	–
	CuCl$_2$	–	+++	+++	+++	–
	Zn(CH$_3$COO)$_2$	+++	+++	+++	+++	–
	ZnCl$_2$	+++	+++	+++	+++	–
	MgCl$_2$	–	–	–	–	–
	FeCl$_2$	+++	+++	+++	+++	–
	FeCl$_3$	+++	+++	+++	+++	–
	3N HCl	–	–	–	–	–

The resulting mixture was evaluated as follows:

   –: the compound was dissolved;

5   +: the mixture was slightly turbid;

   ++: the mixture was significantly turbid;

+++: the mixture was significantly turbid and a precipitation was formed.

     As can be seen from the results of Table 1, a significant

10 turbidity and precipitate formation were observed in each of the phosphate-containing compounds in the presence of zinc, iron (ferric or ferrous), or copper ion.

     When the molar ratio of the betamethasone phosphate to zinc ion and the molar ratio of riboflavin phosphate to zinc ion were

15 varied to examine the amounts of the resulting precipitates, the precipitate formation was most significant for each of the low-molecular weight compounds when the molar ratio with respect to zinc ion was approximately 1.

20 <u>Example 2: Preparation of PLGA/PLA nanoparticles encapsulating</u>
<u>steroids</u>

     Different steroids were dissolved in 100µl water and the resulting solutions were each added to 500µl of a 0.5M aqueous zinc acetate solution or 500µl of a 0.5M aqueous ferrous chloride

solution. Each mixture was centrifuged at 12,000rpm for 5min and the
supernatant was discarded to obtain precipitates in the form of
zinc-steroids or iron-steroids. To the precipitates, 500µl of
acetone, an acetone/acetonitrile mixture, or an acetone/ethanol

5   mixture dissolved 20mg PLGA or PLA (WAKO PURE CHEMICAL INDUSTRIES
LTD.) were added respectively. To each of the resultant solutions,
an aqueous solution of zinc acetate was added and the mixture was
allowed to stand for 2 hours at room temperature. Subsequently, the
solution (or suspension) was added at the rate of 1ml/min via a 27G

10 syringe to a 0.5% aqueous solution of Pluronic F68 (a nonionic high-
molecular weight surfactant) stirring at 400rpm, to give
nanoparticles. The resultant nanoparticles were stirred for 1 to 2
hours at room temperature, and a 0.5M aqueous solution of EDTA (pH8)
was added (0.4 by volume). The mixture was then centrifuged at

15 20,000G for 20min. Following the removal of the supernatant, the
residue was resuspended in water and the suspension was again
centrifuged to wash the nanoparticles. The resulting nanoparticles
were added to a 2N aqueous solution of NaOH to decompose PLGA/PLA,
and the steroid content in the nanoparticles was determined by HPLC.

20 Similarly, the amount of water-insoluble steroid was determined for
the nanoparticles prepared by different method without metal ions.
        Furthermore, precipitates formed by mixing 5mg betamethasone
phosphate with zinc were dissolved in varying volume of acetone and
then encapsulation efficiency of betamethasone phosphate

25 incorporated in the nanoparticles was determined in the same manner
as described above.
        The results are shown in Tables 2 and 3 below.

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Table 2: Encapsulation of steroids into PLGA nanoparticles

Steroids	betamethasone	betamethasone acetate	BDP	BP-Na	BP-Zn
Steroid/nanoparticle (wt%)	0.01	0.15	0.47	0	2.03
Steroids	BP-Fe	DP-Na	DP-Zn	HP-Na	HP-Zn
Steroid/nanoparticle (wt%)	1.15	0	1.15	0	1.05

BDP: betamethasone dipropionate
BP: betamethasone phosphate
DP: dexamethasone phosphate
HP: hydrocortisone phosphate

Table 3 : Effects of the volume of acetone on the encapsulation rate
of betamethasone phosphate into PLGA nanoparticles

Amounts of acetone (μl)	500	700	900	1100	1300	1500
Steroid/nanoparticle (wt%)	*	7.34	4.28	3.46	2.71	1.93

* Data not obtained because of particle aggregation

As shown in Table 2, the use of the precipitates of the
steroid phosphates that were generated through the addition of zinc
or ferrous ion (i.e., BP-Zn, BP-Fe, DP-Zn, and HP-Zn) significantly
increased the encapsulation rate of the respective steroids into
PLGA nanoparticles, as opposed to the cases of the steroid
phosphates provided in the form of sodium salts, each of which
showed substantially no incorporation into the nanoparticles.

Table 3 shows the encapsulation rates of betamethasone
phosphate into PLGA nanoparticles obtained by varying the amount of
the solvent, acetone, while maintaining the amounts of PLGA and
betamethasone phosphate. As can be seen from these results, the
nanoparticles formed aggregates in 500μl or less of acetone. The
particles on the other hand remained stably dispersed in 700μl
acetone while showing a high encapsulation rate of betamethasone
phosphate into the nanoparticles. Although the nanoparticles were
stably dispersed in 700μl or more acetone, the encapsulation rates

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gradually decreased as the amount of acetone was increased.

Example 3: Steroid release profile from PLGA/PLA nanoparticles

5mg betamethasone phosphate was dissolved in 100µl water and

5   the solution was added to 500µl of a 0.5M aqueous solution of zinc
acetate. The mixture was then centrifuged at 12,000rpm for 5min and
the supernatant was discarded to obtain a zinc-steroid precipitate.
To the precipitate, 500µl of acetone dissolved 20mg of PLGAs or PLAs
with different molecular weights was added. The solution was allowed

10   to stand for 2 hours at room temperature and was subsequently added,
at a rate of 1ml/min with a 27G syringe, to a 0.5% suspension of
either Pluronic F68 (a nonionic high-molecular weight surfactant) or
lecithin that had been stirred at 400rpm. The resulting
nanoparticles were stirred for 1 to 2 hours at room temperature.

15   Following the addition of EDTA, the nanoparticles were subjected to
ultrafiltration on Centriprep YM-10 (Amicon) for concentration and
washing. The nanoparticles were then suspended in a mixture of FBS
(fetal bovine serum)/PBS (v/v=1) at a 500µg/mL PLGA concentration
and, after a predetermined period of time, a 0.5M aqueous solution

20   of EDTA (pH8) was added (0.4 by volume). The suspension was then
centrifuged at 20,000G for 30min and the supernatant was discarded.
The residue was resuspended in water and the suspension was again
centrifuged to wash the nanoparticles. The resulting nanoparticles
were added to a 2N aqueous solution of NaOH to hydrolyze PLGA/PLA,

25   and the steroid content in the nanoparticles was determined by HPLC.
As a control, nanoparticles encapsulating BDP (betamethasone
dipropionate), a hydrophobic steroid, were prepared according to a
method proposed by the present inventors in a previous patent
application (Japanese Patent Application No. 2002-159190). The

30   amount of the encapsulated steroid was determined in the same manner.
The results are shown in Table 4 below.

Table 4: Release of betamethasone from nanoparticles

PLGA/PLA	Cumulative betamethasone released (%)						
	5hrs	Day1	Day2	Day4	Day8	Day11	Day20
PLA[*1] (Mw 14000)	27	53	64	79	97	98	100
PLGA[*2] (Mw 8000)	0	17	29	35	60	70	93
PLGA[*2] (Mw 13000)	0	11	18	34	47	53	62
PLA[*2] (Mw 9000)	0	12	13	25	28	30	38
PLA[*2] (Mw 14000)	0	3	4	8	10	14	31

*1: Nanoparticles prepared according to the method described in Japanese Patent Application No. 2002-159190
*2: Nanoparticles prepared according to the method of the present invention

It was demonstrated that the nanoparticles encapsulating BDP (betamethasone dipropionate), a hydrophobic steroid, and prepared according to the method previously proposed by the present inventors (Japanese Patent Application No. 2002-159190) released a significant amounts of betamethasone at an early stage with approximately 90% or more of betamethasone having been released after 6 days. In contrast, the nanoparticles prepared according to the method of the present invention, in which the steroid's initial bursting release is significantly reduced, released the steroid in a more gradual manner and were able to release it over an extended period of time.

It has also been demonstrated that the nanoparticles made of PLGA or PLA with small molecular weights tend to release the steroid at an earlier stage and that the nanoparticles made of PLGA tend to release the steroid earlier than those made of PLA.

Example 4: Release profile of steroids from nanoparticles internalized by macrophages

Macrophages were collected from the abdominal cavities of mice that had been stimulated by intraperitoneal administration of 1.5ml of 10% proteose peptone. The cells were inoculated at 6×10^5

cells/12 wells and were cultured overnight in Macrophage-SFM medium
(Gibco). Subsequently, the culture medium was replaced, and the PLGA
or PLA nanoparticles prepared according to the procedures described
in Example 3 were added. The cells were incubated at 37°C for

5   another 2 hours. Subsequently, the cells were washed 8 times with
PBS and the medium, and the amount of betamethasone in the medium
was determined at pre-determined intervals by ELISA method.

As a control, nanoparticles encapsulating BDP (betamethasone
dipropionate), a hydrophobic steroid, were prepared according to a

10   method previously proposed by the present inventors (Japanese Patent
Application No. 2002-159190) and were also added to the cells.

The results are shown in Table 5 below.

Table 5: Release profiles of betamethasone from macrophages

15   internalizing nanoparticles

	Cumulative betamethasone released (%)							
	2hrs	4hrs	10hrs	Day1	Day2	Day3	Day5	Day7
Control nanoparticles[*1]	26	42	68	86	96	97	98	99
Nanoparticles of the present invention[*2]	3	4	11	27	64	77	89	96

*1: Nanoparticles prepared using PLA (MW 14,000) (Japanese Patent
    Application No. 2002-159190)
*2: Nanoparticles prepared using PLGA (MW 8,000)

20   It was demonstrated that the nanoparticles encapsulating BDP
(betamethasone dipropionate), a hydrophobic steroid, and prepared
according to the method previously proposed by the present inventors
(Japanese Patent Application No. 2002-159190) had released most of
betamethasone as early as after 2 days. In contrast, the

25   nanoparticles prepared according to the method of the present
invention showed a nearly linear release profile during the first 2
to 3-day period and continued to gradually release betamethasone for
a succeeding period.

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Example 5: Evaluation of dispersion stability of the nanoparticles

     The acetone solutions prepared according to the procedures described in Example 3 were added dropwise to aqueous solutions of different surfactants to obtain nanoparticles. The resulting

5 nanoparticles were concentrated, washed, purified, and were then freeze-dried in sucrose solutions of varying concentrations. The freeze-dried nanoparticles were resuspended in water and particle sizes of the particles were measured using a light-scattering photometer.

10   All of the nanoparticles prepared by using aqueous solutions of different surfactants, namely, lecithin, polyoxyethylene polyoxypropylene glycols, and polysorbates, had substantially the same particle size. No significant differences were observed among the surfactants in the size and the dispersion stability of the

15 nanoparticles, and in the encapsulation rate of betamethasone phosphate even when the concentrations of the surfactants were varied in the range from 0.01 to 1%.

     In comparison, the nanoparticles prepared with a polyvinylalcohol solution were larger in size than those prepared

20 with other surfactants and had a low encapsulation rate of betamethasone phosphate. It was also shown that the re-dispersibility of the freeze-dried nanoparticles by adding sucrose in an amount (by weight) more than 5 times the amount of the nanoparticles prior to freeze-drying the nanoparticles.

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Example 6: Accumulation of nanoparticles in inflammatory sites

     Inflammation was induced by injecting 100µl physiological saline containing 1% carrageenin in the left hind paw of male Lewis rats. After 4 hours, single dosages of rhodamine-encapsulating

30 nanoparticles of two different sizes (200nm and 500nm) were injected into a tail vein. 2 hours after administration, the resultant leg edema was cut and cryostat sections were prepared. The tissue samples were observed with fluorescence microscopy.

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As controls, one group was administered with physiological saline and another group with rhodamine alone.

The intensity of fluorescence observed in tissue sections was significantly higher in the group given the 200nm nanoparticles than in the control group given physiological saline alone, indicating significant accumulation of the nanoparticles in the inflammatory sites.

No significant accumulation of the nanoparticles was observed in the group given rhodamine alone or the group administered with the 500nm nanoparticles.

Example 7: Activity to suppress adjuvant-induced arthritis

Arthritis was induced in 7-week old Lewis rats, weighing 130 to 160g and preconditioned for one week, by injecting, under ether anesthesia, 50µl of incomplete Freund's adjuvant solution (DIFCO) containing 6mg/mL M. Butyricum Desiccated (DIFCO) into the left hind paw. The animals were divided into groups so that there are no significant differences between the groups in terms of the volume of the left hind leg of the animals. 14 days after administration of M. Butyricum, a single dose of PLA nanoparticles encapsulating betamethasone phosphate was administered intravenously to one group.

As controls, single doses of betamethasone phosphate and phosphate-buffered saline (PBS) were subcutaneously administered to respective groups of rats and a single dose of limethason (MITSUBISHI PHARMA) was intravenously administered to another group.

The ability of the nanoparticles to suppress inflammation was analyzed by measuring the volume of the left hind legs before and 7 days after the administration of the drug using water displacement technique.

The results are shown in Table 6 below.

Table 6: Abilities of the nanoparticles to suppress adjuvant-induced
arthritis

Groups	Inflammation rate (%) after administration (# of days)[3]						
	1	2	3	4	5	6	7
Nanoparticles of the present invention[1]	69	68.7	68.3	69	70.3	70.8	71.3
Limethason [2]	66.9	72	79.2	78.5	80	79	—
Betamethasone phosphate(300µg)	68.3	76.5	79.2	81.7	88	—	84.8
Betamethasone phosphate(100µg)	78.4	80	82.8	85.4	84.2	83	81.7
Physiological saline	100.8	98.1	98	96.7	96	95.5	96.2

*1: Betamethasone phosphate-encapsulating nanoparticles were prepared using
PLA (MW 14000). Nanoparticles were given in an amount corresponding to
100µg Betamethasone phosphate.

*2: Given in an amount corresponding to 100µg dexamethasone phosphate.

*3: Inflammation rate was calculated by the following equation:
Inflammation rate (%) = (measured leg volume – leg volume of normal rat
un-injected adjuvant)/(leg volume before steroid administration – leg
volume of normal rat un-injected adjuvant) x 100

As shown in Table 6, an anti-inflammatory effect comparable to
that observed with the use of three times as much of the
betamethasone phosphate was seen in the group administered with
limethason, an anti-inflammatory agent already in clinical use, as
early as 1 day after administration. As with the case where
betamethasone phosphate alone was administered, the anti-
inflammatory effect of limethason was gradually lost over time. In
comparison, the PLA nanoparticles of the present invention
encapsulating betamethasone phosphate exhibited, as early as 1 day
after administration, a high anti-inflammatory effect comparable to
that observed with limethason and continued to exhibit a strong
effect over a succeeding 7-day period.

Example 8: Preparation of PLGA/PLA nanoparticles encapsulating PGE$_1$

1mg of PGE$_1$ was dissolved in 20µl ethanol and the solution was
added to an 80µl 0.5M aqueous solution of ferrous (or ferric)
chloride. The mixture was then centrifuged at 12,000rpm for 5min and

the supernatant was removed to obtain an iron-PGE$_1$ precipitate. To
this precipitate, PLGA (WAKO PURE CHEMICAL INDUSTRIES, LTD.) or PLA
(WAKO PURE CHEMICAL INDUSTRIES, LTD.) in acetone was added. An
aqueous solution of zinc acetate was further added and the solution

5   was allowed to stand for 2 hours at room temperature. Using a 27G
syringe, the solution (or suspension) was subsequently added, at a
rate of 1ml/min, to a 0.5% suspension of either Pluronic F68 (a
nonionic high-molecular weight surfactant) or lecithin that had been
pre-stirred at 400rpm. The resulting nanoparticles were stirred for

10   1 to 2 hours at room temperature and a 0.5M aqueous solution of EDTA
(pH8) was added (0.4 by volume). The suspension was then centrifuged
at 20,000G for 20min and the supernatant was discarded. The residue
was resuspended in water and the suspension was again centrifuged to
wash the nanoparticles. The resulting nanoparticles were dissolved

15   in acetonitrile, followed by dilution with PBS. The amount of PGE$_1$
was then determined by ELISA method.

       As described in Example 4, macrophages were allowed to uptake
the PGE$_1$-encapsulating PLGA nanoparticles and the amount of PGE$_1$
contained in the medium was determined at intervals by ELISA.

20   The results are shown in Table 7 below.

Table 7: Release profile of PGE$_1$ from macrophages internalizing
nanoparticles

	Cumulative PGE$_1$ released (%)								
	2hrs	5hrs	10hrs	Day1	Day2	Day3	Day4	Day6	Day8
PGE$_1$-encapsulating nanoparticles[1]	22	42	60	75	90	95	98	99	100

[1]: Nanoparticles prepared using PLGA (MW 8,000)

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       The encapsulation rate of PGE$_1$ into the PLGA nanoparticles was
approximately 0.1 to 1% by weight. As can also be seen from the
results shown in Table 7, PGE$_1$ was continuously released from the
nanoparticles for 8 days although the release profile was not as

30   good as that for betamethasone phosphate, a steroidal anti-

inflammatory agent.

INDUSTRIAL APPLICABILITY

As set forth, the present invention provides intravenous PLGA
or PLA nanoparticles that can encapsulate sufficient amounts of low-
molecular weight, water-soluble and non-peptide drugs are less
likely to burst at an early stage of administration, and are capable
of releasing the drug for a prolonged period of time.

The intravenous nanoparticles of the present invention can be
used to target various inflammatory sites, vascular lesion sites,
infectious sites, and malignant tumor tissues and effectively
accumulate in such sites or tissues where the encapsulated low-
molecular weight, water-soluble and non-peptide drugs are released
over time to exhibit their biological activities for a prolonged
period of time. The potential medical impact that the nanoparticles
of the present invention can bring about is thus significant.